Rapid proteolysis of IκB-α is necessary for activation of transcription factor NF-κB

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Inducible gene expression in eukaryotes is mainly controlled by the activity of transcriptional activator proteins, such as NF-κB (refs 1–3), a factor activated upon treatment of cells with phorbol esters, lipopolysaccharide[4], interleukin-1 and tumour necrosis factor-α[5]. Activation of NF-κB involves release of the inhibitory subunit IκB from a cytoplasmic complex with the DNA-binding subunits Rel-A (formerly p65) and p50 (refs 6, 7). Cell-free experiments have suggested that protein kinase C and other kinases transfer phosphoryl groups onto IκB causing release of IκB and subsequent activation of NF-κB[8–10]. Here we report that IκB-α (formerly MAD-3)[11] is degraded in cells after stimulation with phorbol ester, interleukin-1, lipopolysaccharide and tumour necrosis factor-α, an event coincident with the appearance of active NF-κB. Treatment of cells with various protease inhibitors or an antioxidant completely prevented the inducible decay of IκB-α as well as the activation of NF-κB. Our findings suggest that the activation of NF-κB relies on an inducible degradation of IκB-α through a cytoplasmic, chymotrypsin-like protease. In intact cells, phosphorylation of IκB-α is apparently not sufficient for activation of NF-κB.

We investigated the fate of IκB-α after treatments of cells with various NF-κB-activating stimuli. In extracts from unstimulated 70Z/3 pre-B cells, IκB-α-specific IgG detected a single 38K band on western blots (Fig. 1*a*, lane 1) which was not seen with the second antibody alone (data not shown). Between 2 and 5 min after the addition of phorbol 12-myristate 13-acetate (PMA) to cell cultures, IκB-α almost completely disappeared from cells (Fig. 1*a*, compare lanes 3 and 4). The specific immunoreactivity reappeared after 40 min (Fig. 1*a*, lane 7). The disappearance of IκB-α coincided with the appearance of NF-κB DNA-binding activity in total cell extracts (Fig. 1*b*, compare lanes 2 and 3), suggesting a causal relationship between the two events. Interleukin (IL-1β), lipopolysaccharide (LPS) and tumour necrosis factor-α (TNF-α) all induced a decay of IκB-α in 70Z/3 or HeLa cells (Fig. 1*c*), showing that distinct stimuli induced the same reaction. Although most of the IκB-α had already decayed 5 min after stimulation with PMA, TNF-α and IL-1β, it took more than 15 min before there was a rapid decay of the inhibitor in LPS-stimulated 70Z/3 cells. Despite these kinetic differences, the depletion of IκB-α was in each case coincident with the

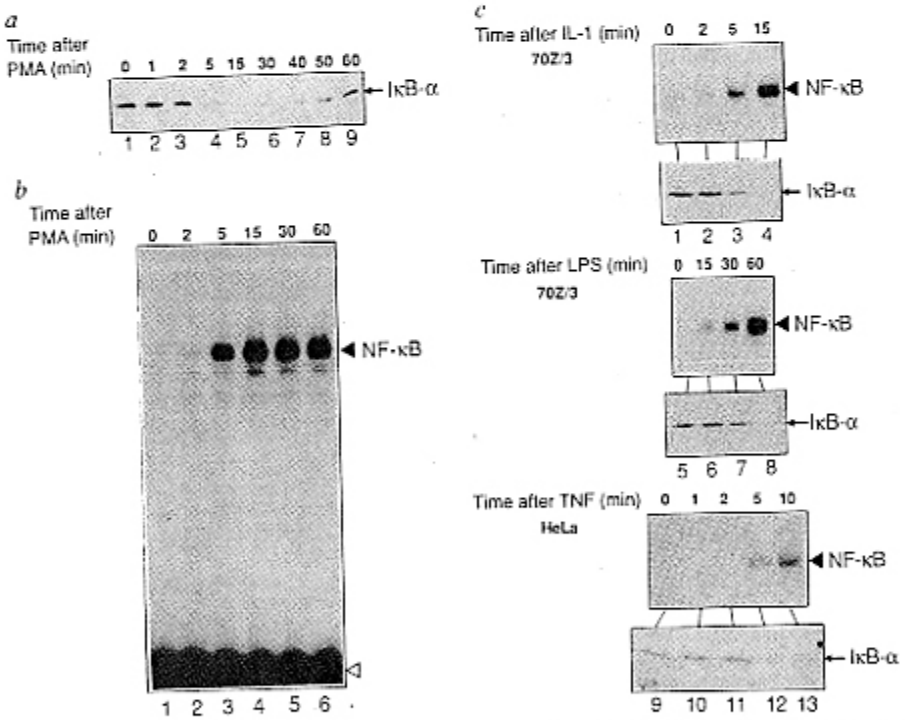

FIG. 1 The fate of IκB-α in stimulated cells. *a*, The effect of PMA on IκB-α immunoreactivity in 70Z/3 cells. Cells were treated with PMA for the indicated periods of time (lanes 2–9). Proteins in total cell extracts were separated by SDS–PAGE and transferred onto a membrane filter followed by western blot analysis using anti-IκB-α IgG and decoration with peroxidase-labelled anti-rabbit IgG. The arrow indicates the position of IκB-α. A section of a western blot is shown. *b*, The effect of PMA on the DNA-binding activity of NF-κB. Total cell extracts from control (lane 1) and PMA-treated cells (lanes 2–6) were incubated with a ^{32}P-labelled DNA probe encompassing the NF-κB-binding motif from the mouse κ light chain enhancer[4] followed by analysis of DNA-binding activities using electrophoretic mobility shift assay (EMSA). A fluorogram of a native gel is shown. *c*, The effect of IL-1β, LPS and TNF on the activation of NF-κB and stability of IκB-α. 70Z/3 cells (upper and middle panels) or HeLa cells (lower panel) were treated with IL-1β (lanes 2–4), LPS (lanes 6–8) or TNF-α (lanes 10–13) for the indicated periods of time followed by analysis of total cell extracts for NF-κB activity and IκB-α immunoreactivity. Sections of western blots and of fluorograms from native gels are shown. The position of the NF-κB-NDNA complex is shown by a filled arrowhead, the position of uncomplexed DNA probe by an open arrowhead and the position of the IκB-α band by an arrow.

METHODS. 70Z/3 cells were cultured as described elsewhere[4], and treated with 50 ng ml^{-1} PMA (Sigma), 50 U ml^{-1} IL-1β (Boehringer–Mannheim) or 15 μg ml^{-1} LPS (Sigma). HeLa cells were cultured in DMEM supplemented with 10% fetal calf serum and 1% L-glutamine, and treated with 200 U ml^{-1} recombinant human TNF-α (Genzyme). Stimulation was stopped by cooling on ice and immediate centrifugation of cells for 5 s in an Eppendorf microfuge. Cell pellets (10^6 cells) were lysed with a high-salt extraction buffer[7]. Supernatants of lysates were used for analysis by both western blotting and EMSA, as described[27,28]. Specificity of the protein–DNA complex was verified by immunoreactivity with a polyclonal antibody specific for Rel-A (data not shown). Two milligrams of 6×His-tagged, purified human IκB-α produced in *Escherichia coli*[27] were coupled to 0.5 ml cyanogen bromide-activated sepharose 4CL-B (Pharmacia), according to the instruction of the manufacturer. Specific IgG in 2 ml rabbit antiserum raised against IκB-α[27] was affinity-purified[28] on IκB-α-sepharose. After extensive washes with phosphate-buffered saline and 2 column vols 0.1 M glycine–HCl, pH 2.7, specific antibodies were eluted with 2 vols 4 M guanidinium hydrochloride. The eluate was extensively dialysed against Tris-buffered saline and used for western blotting at a dilution of 1:100 in blocking buffer[28].

appearance of NF-κB activity. In contrast to another study[12], no transient change in the electrophoretic mobility of IκB-α was apparent after induction with the various stimuli, even when phosphatase inhibitors were included in the lysis buffer (data not shown). Moreover, we could not detect any breakdown products of IκB-α (data not shown). Because a polyclonal antibody was used, it is unlikely that only one epitope of IκB-α was lost or modified upon stimulation.

The protein synthesis inhibitor cycloheximide (CHX) activates NF-κB in 70Z/3 cells[4]. Following a 1 h treatment of 70Z/3 cells with CHX, the DNA binding of NF-κB was induced only weakly (Fig. 2*a*, lane 1, upper panel), and significant levels of IκB-α were still present (lower panel). This shows that interference with the normal turnover rate of IκB-α is not sufficient for a rapid activation of NF-κB. When CHX-treated cells were stimulated with PMA, a rapid decay of IκB-α and further induction of NF-κB binding activity were observed with kinetics indistinguishable from those observed in the absence of CHX (compare Fig. 2*a* with Fig. 1*a*). But CHX prevented the reappearance of IκB-α seen after a 40 min treatment with PMA alone (compare Fig. 2*a* with Fig. 1*a*), consistent with the finding that IκB-α is newly synthesized under transcriptional control by NF-κB[12–14]. We determined a rough half-life of 138 min for IκB-α in CHX-treated 70Z/3 cells (Fig. 2*b*). If protein synthesis-arrested cells were stimulated with PMA, the half-life of IκB-α was reduced to only 1.5 min during the phase of its fastest decay, showing that PMA induced a two orders of magnitude decrease in the half-life of IκB-α.

The functional significance of the inducible decay of IκB-α for activation of NF-κB was tested by exposure of cell cultures to various protease inhibitors. Six distinct serine protease inhibitors with chymotrypsin-like specificity efficiently prevent the induction of NF-κB DNA-binding activity in response to PMA and TNF-α (Table 1). Constitutive DNA-binding activities, including Oct-1, were not affected and no significant cell death was observed by a dye-exclusion assay and phase-contrast microscopy at inhibitor concentrations preventing NF-κB activation. Leupeptin, antipain and the trypsin inhibitor tosyl-Lys-methylester were not effective, even at high concentrations (T.M. and M.K., manuscript in preparation).

TABLE 1 The effect of various protease inhibitors on the activation of NF-κB

Protease inhibitor	IC_{90} (μM) Jurkat	70Z/3
Tosyl-Phe-chloromethylketone (TPCK)	20	20
Benzyloxycarbonyl-Leu-Tyr-chloromethylketone (zLYCK)	10	10
Tosyl-Lys-chloromethylketone (TLCK)	100	100
3,4-Dichloroisocoumarin	20	ND
N-Benzoyl-L-Tyr-ethylester (BTEE)	1,000	ND
N-Acetyl-DL-Phe-β-naphthylester (APNE)	200	200

Jurkat and 70Z/3 cell cultures were incubated with the listed compounds at various concentrations for 30 min and than stimulated by either TNF-α (Jurkat cells) or PMA (70Z/3). Total cell or nuclear extracts were prepared and analysed for NF-κB activity using EMSA. IC_{90}, 90% inhibitory concentration; ND, not determined. Jurkat T cells were cultured as described[15]. All compounds (Sigma) were dissolved in DMSO and cells pretreated at various concentrations for 30 min. EMSA was done as described[28].

The effect of tosyl-Phe-chloromethylketone (TPCK) was investigated in more detail. Treatment of cells with 25 μM TPCK fully inhibited the induction of NF-κB activity (Fig. 3*a*, upper panels) as well as the decay of IκB-α in response to PMA (lower panel). The half-maximal inhibitory concentration (IC_{50}) of TPCK was about 8 μM. The protease inhibitor prevented the activation of NF-κB by IL-1β and LPS in 70Z/3 cells (Fig. 3*b*, lanes 6 and 7) and by TNF-α in various other cell lines (T.M. and M.K., manuscript in preparation). TPCK did not strongly interfere with NF-κB activity when added after stimulation with PMA, IL-1 or LPS but seemed to arrest a further activation of the factor (Fig. 3*b*, lanes 10–12). It is unlikely that TPCK acted on protein kinase C (PKC) because IL-1 and TNF-α activate NF-κB independently of PMA-inducible PKC isoenzymes[15,16]. Furthermore, TPCK did not interfere with early signalling events of the TNF receptor[17] (T.M. and M.K., manuscript in preparation). TLCK, an inhibitor of trypsin-like serine proteases, which is highly related in structure and chemical activity to TPCK,

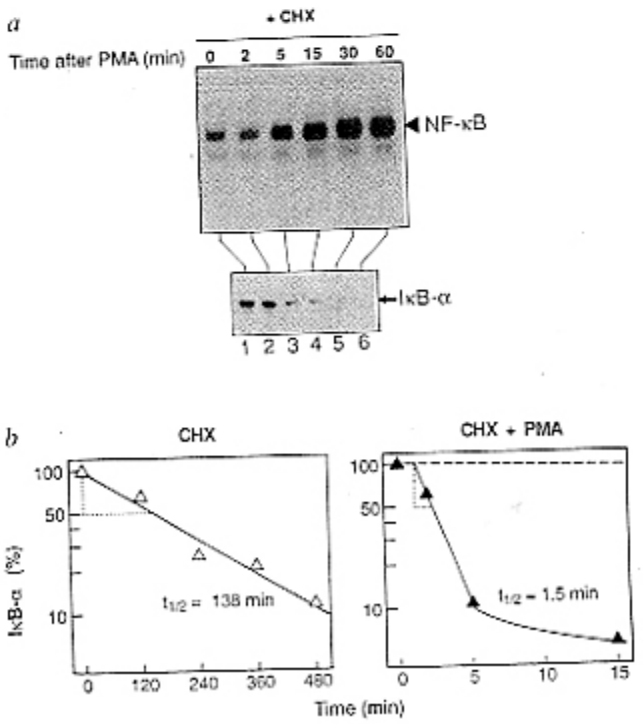

FIG. 2 The effect of a protein synthesis inhibitor on the stability of IκB-α and the activation of NF-κB. *a*, 70Z/3 cells were treated with cycloheximide (CHX) for 1 h followed by stimulation with PMA for the indicated periods of time. Total cell extracts were assayed for NF-κB activity by EMSA (upper panel). A section of a fluorogram from a native gel is shown with a filled arrowhead indicating the position of the NF-κB-DNA complex. Aliquots of the cell extracts were assayed for IκB-α by western blotting (lower panel). The position of the IκB-α band is indicated by an arrow. *b*, Half-life of IκB-α in protein synthesis-arrested cells. Cells were treated with CHX alone (left panel) or, after a 1 h treatment with CHX, induced with PMA for the indicated times (right panel). Total cell extracts of equal protein content were analysed by western blotting followed by densitometric quantitation of the 38K IκB-α band in fluorograms. The amounts of IκB-α detected with CHX treatment alone (left panel; open triangles) and after PMA stimulation (right panel, filled triangles) are shown on a half-logarithmic plot. Dotted lines indicate the portion of the slopes used for estimation of $t_{1/2}$ values. The dashed line in the right panel indicates the decay of IκB-α in the absence of PMA, as determined in the left panel.
METHODS. 70Z/3 cells were treated with 25 μg ml^{-1} cycloheximide (Sigma). At 10 μg ml^{-1}, protein synthesis is inhibited to 90% in 70Z/3 cells[29]. Cell culture, extract preparation, EMSA, SDS–PAGE and western blotting were as described in the legend to Fig. 1. Densitometric scanning was done with a Howtek Scanmaster 3 and data analysed by the software Quantity One Version 2.2.

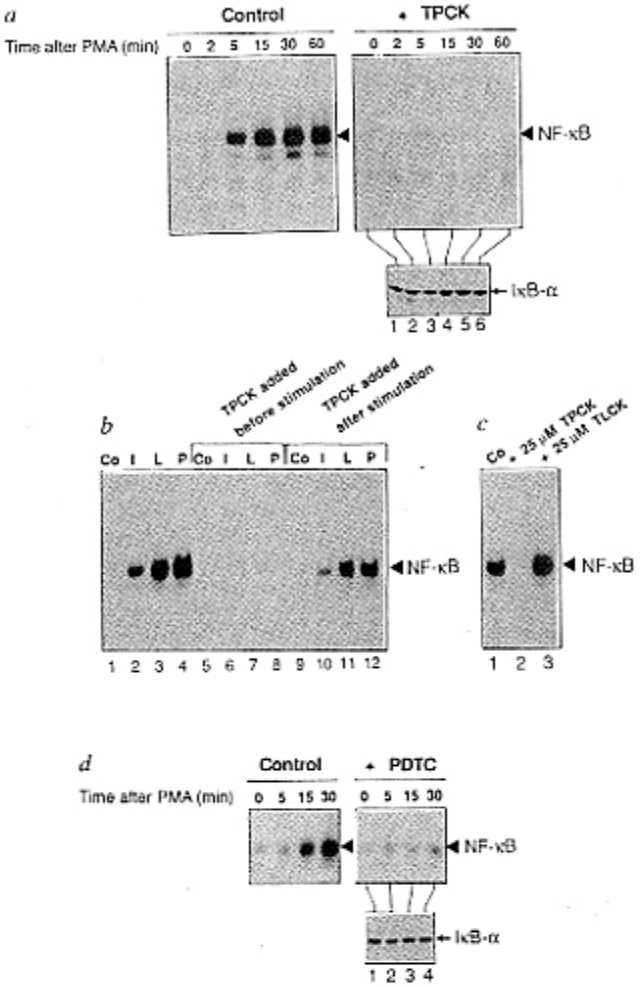

FIG. 3 The effect of various inhibitors on the activation of NF-κB and stability of IκB-α. *a*, The effect of TPCK. 70Z/3 cells were stimulated with PMA for the indicated times in the absence (left panel) or presence of 25 μM TPCK (1 h pretreatment). Cell extracts were analysed for NF-κB activity by EMSA (upper panels) and for IκB-α levels by western blotting (lower panel). An arrow indicates the position of the 38K IκB-α band in western blots. The faint lower band is nonspecific because its abundance was strongly reduced when affinity-purified antibody was used. The position of the NF-κB–DNA complex in the sections of fluorograms is indicated by filled arrowheads. *b*, The effect of TPCK on the activation of NF-κB by IL-1β, LPS and PMA in 70Z/3 cells. Co, Control cells. Cells were treated with TPCK 10 min before stimulation (lanes 6–8) or, for 10 min, following treatments of cells for 30 min with IL-1 (L) and PMA (P), and for 60 min with LPS (L) (lanes 10–12). Total cell extracts from control (lanes 1–4) and TPCK-treated cells (lanes 5–12) were analysed for NF-κB activity by EMSA. *c*, The effect of TLCK on the activation of NF-κB by PMA. 70Z/3 cells were left untreated (lane 1) or treated for 10 min with either 25 μM TPCK (lane 2) or 25 μM TLCK (lane 3) followed by addition of PMA. Cell extracts were analysed by EMSA for NF-κB activity. *d*, The effect of the antioxidant PDTC. Cells were treated for the indicated periods of time with PMA in the absence or presence of 100 μM PDTC added 1 h before stimulation. Cell extracts were analysed for NF-κB activity by EMSA (upper panels) and for IκB-α levels by western blotting (lower panel).

METHODS. Cell culture, extract preparation, EMSA, SDS–PAGE and western blotting were as described in the legend to Fig. 1. 70Z/3 cells were pretreated with 25 μM TPCK or TLCK (Sigma) dissolved in dimethylsulphoxide (DMSO). Control cultures received an equivalent amount of DMSO. Cell cultures were treated with the ammonium salt of PDTC (Sigma), as described in detail elsewhere[20].

could not prevent activation of NF-κB at a concentration of 25 μM (Fig. 3*c*, lane 3), but only at 100 μM (Table 1). The pharmacological data strongly support the requirement of proteolytic degradation of IκB-α for the activation of NF-κB. The inhibitor profile suggests the involvement of a serine protease with chymotrypsin-like specificity, which we will refer to as IκB-α protease. We cannot rule out that the protease inhibitors interfered with another proteolytic step located upstream from the IκB-α protease. But this appears unlikely in view of the diversity of NF-κB activators, which might have very few upstream pathways in common.

Reactive oxygen intermediates (ROIs) seem to play a role as messengers in the activation of NF-κB by many inducing conditions[18–20]. A potent antioxidant inhibitor of NF-κB activation is pyrrolidinedithiocarbamate (PDTC)[20]. No significant decay of IκB-α or activation of NF-κB was seen after PMA stimulation in 70Z/3 cells pre-treated with 100 μM PDTC (Fig. 3*d*), suggesting the proteolysis of IκB-α is controlled by ROIs. PDTC does not interfere with the PMA-induced membrane association and kinase activity of PKC[21], which argues against a direct phosphorylation of IκB-α by PKC in intact cells.

An inducible degradation of IκB-α could have been controlled by different mechanisms. First, a covalent modification could have tagged IκB-α for degradation by a constitutive protease. Second, the IκB-α-protease was newly activated. The protease (or a protease inhibitor) could be a direct target for protein kinases or ROIs. Third, both tagging of the substrate as well as induction of protease activity were required. A controlled proteolytic degradation of IκB-α is exquisitely suited for an irreversible activation of NF-κB. The only mechanism to re-inhibit activated NF-κB is then through newly synthesized IκB-α. This would explain why nuclear NF-κB is inactivated in PMA-treated 70Z/3 cells depending on new protein synthesis[22], and why IκB-α can enter the nucleus[23] and remove NF-κB from DNA[24]. An important open question is whether the IκB-α-protease can attack IκB-α in the cytoplasmic complex with NF-κB or only after its release from NF-κB. A selective degradation of a subunit within a transcriptional regulator was observed with the α2 repressor from yeast[25]. Is phosphorylation required for the release of IκB-α from NF-κB? No data have yet been obtained from intact cells in strong support for an inducible phosphorylation of IκB-α, although a constitutive phosphorylation of IκB-α was observed (I.A. and Y.B.-N., unpublished data). Our data can only be reconciled with *in vitro* kinase data[8–10] on the assumption that degradation is required in intact cells to eliminate phosphorylation-released IκB-α rapidly. This would prevent IκB-α, once it is dephosphorylated, from re-inhibiting NF-κB. But given the strong effect of protease inhibitors, IκB-α phosphorylation alone appears insufficient for activation of NF-κB in intact cells. NF-κB is critically involved in many pathological events including progression of AIDS, the acute phase response and the activation of immune and endothelial cells during toxic shock, allograft rejection, ultraviolet and radiation responses[1,26]. We thus expect specific inhibitors of the IκB-α protease to be effective as novel anti-inflammatory and immunosuppressive drugs. □

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Efficient catalysis of disulphide bond rearrangements by protein disulphide isomerase

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PROTEIN disulphide isomerase (PDI)[1,2] is a highly abundant and ubiquitous eukaryotic protein that is essential for viability in yeast[3,4]. Although PDI is thought to catalyse disulphide bond formation and isomerization during protein biosynthesis, PDI has been found previously to have only moderate effects (~25-fold) on the rate of oxidative folding of proteins *in vitro*. In addition, PDI has been implicated in several apparently unrelated cellular functions[3]. For example, PDI is the β-subunit of prolyl 4-hydroxylase[5] and is part of the trigylceride transfer complex[6]. The oxidative folding of bovine pancreatic trypsin inhibitor (BPTI) is slow and inefficient *in vitro*[7–11]. Here we report that PDI increases by a factor of 3,000–6,000 the rates of folding of kinetically trapped BPTI folding intermediates, in which native structure impedes disulphide bond formation. By contrast, PDI has only small effects on the rate of disulphide bond formation in intermediates that are oxidized readily in the absence of PDI. These results suggest that an important function of PDI is to catalyse disulphide bond formation and rearrangements within kinetically trapped, structured folding intermediates.

The best characterized disulphide folding pathway of a protein is for the *in vitro* folding of BPTI[7–11] (Fig. 1*a*, *b*). During folding at neutral pH, BPTI accumulates rapidly as one of two intermediates. These intermediates, termed N* and N′, both contain two native disulphide bonds, [5–55; 14–38] and [30–51; 14–38], respectively. About half of the molecules become trapped as N*, which is a dead-end intermediate that is stable for weeks. N′ rearranges slowly (hours) to N*, and also to a third native two-disulphide intermediate ([30–51; 5–55]), termed N_{SH}^{SH}, which is oxidized readily to native BPTI (N).

Formation of the final native disulphide bond in N* and N′ is hindered by native structure in these intermediates which constrains and buries the remaining free thiols[8,9]. Recently, a naturally occurring amino-terminal pro- region (ref. 12; J. Li, S. Olson and D. A. Walz, personal communication) was shown to increase both the rate and yield of folding of BPTI[11]. Nonetheless, even in this model of pro-BPTI, folding is slow and ~25% of the molecules accumulate as the dead-end intermediate N*.

TABLE 1 Kinetic parameters for catalysis by PDI

Transition	k_{cat} (min^{-1})	K_m (μM)	k_{uncat}† (min^{-1})	Fold ‡ acceleration
N′→N	5	7	1.4×10^{-3}	3,500
N*→N	0.3	30	5×10^{-5}	6,000
proN*→proN	0.9	35	2×10^{-4}	4,500

BPTI folding experiments were done at 25 °C, 2.0 mM GSH, 0.5 mM GSSG, pH 7.3.

† k_{uncat} is the rate in the absence of PDI. The values for N* and proN* are reliable only to within a factor of 2, owing to difficulties associated with measuring a rate over a period of several days under anaerobic conditions[10]. Michaelis–Menten parameters (K_m and k_{cat}) for the catalysis of N′, N* and proN* were determined by measuring the initial rate of formation of native BPTI or pro-BPTI by HPLC. Initial velocity measurements were made at five or six substrate concentrations that ranged from 1.5 μM to 100 μM for N′ and from 5 μM to 150 μM for N* and proN*. The concentration of PDI used ranged from 0.17–0.7 μM. Kinetic constants were calculated from Lineweaver–Burk plots. The initial velocity measurements were made at times in which less than one third of the starting material had rearranged. The values of k_{cat}/K_m were confirmed by measuring the rate of formation of native BPTI or Pro-BPTI for the entire time course of the reactions at initial substrate concentrations at least fivefold below K_m. Catalysis of proN′ does not appear to follow Michaelis–Menten kinetics; the rate of formation of native BPTI is not constant during initial rate measurements. In addition, several well populated Intermediates are present and the level of these intermediates, relative to proN′, increases throughout most of the reaction. Qualitatively, however, the rate of folding of proN′ in the presence of PDI is comparable to that of N′ from mature BPTI.

‡ Fold acceleration is k_{cat}/k_{uncat}. Under optimal redox conditions (1.0 mM GSH, 0.2 mM GSSG, pH 8.0, 25 °C) k_{uncat} for the regeneration of reduced RNase A is 0.02 min^{-1} and k_{cat} is 0.46 min^{-1} (ref. 16).

Earlier studies[13,14] found that PDI increased the rate of oxidation and reduction of BPTI in the presence of dithiothreitol, but had little effect on folding when the physiological[15] redox reagent glutathione was used. In those studies, however, N* was not distinguished from native BPTI (N). In addition, folding was done in the absence of a reducing agent. Subsequently it has been shown, at least for the folding of RNase A in the presence of glutathione, that catalysis of disulphide bond formation by PDI requires a redox buffer that contains reduced and oxidized components[16].

We show here that PDI increases dramatically both the yield and rate of formation of native BPTI in a physiological redox buffer[15] (Fig. 1*c*, *d*). The rate of formation of the two kinetically trapped native intermediates, N′ and N*, is increased moderately (~3-fold) by PDI. The striking feature of the PDI-catalysed folding reaction is that the N′ and N* intermediates appear to be converted readily to native BPTI (N).

Working with purified, reversibly trapped species[9], we examined directly the effect of PDI on the folding of N′ and N*. In